

A METHOD OF MEASURING THE DURATION OF ADEQUATE IMMUNE MEMORY IN  
COMPANION ANIMALS

Background of the Invention

5 The purpose of vaccination is two fold: to protect the individual from disease and to  
maintain a large enough number of immune individuals in the population so that the disease  
is not readily transmitted. As long as there is risk of infection, a threshold level of immune  
individuals is required to maintain herd immunity. If the number of immune individuals in the  
population decreases below this threshold, either due to waning of vaccine protection or non-  
vaccination, disease outbreaks can occur. Thus, an important parameter of a vaccine is how  
10 long it is able to protect the animal from infection.

15 Currently, because little to no duration of immunity information is available for most  
animal vaccines, annual vaccination is recommended by most vaccine labels and  
veterinarians (Schultz R.D. *Veterinary Medicine* 93:233-254 (1998)). These annual vaccine  
administration recommendations had been made as early as 1961 and have since been  
established as the standard practice by veterinary organizations. But recent information  
suggests that many vaccines provide effective immunity in companion animals for years;  
therefore, annual vaccine administration may not be necessary (Macy D.W. *J. Am. Vet. Med.*  
*Assoc.* 207:421-425 (1995)).

20 Until recently, most vaccines were evaluated for duration of immunity for only a few  
weeks or months but not necessarily the full vaccination interval touted on the label.  
Recently, USDA rules have been expanded to require that newly approved novel veterinary  
vaccines be shown to induce the minimum duration of immunity stated on the product label,  
but, manufacturers are not required to establish maximum duration of immunity provided by  
the vaccine (1998 Report of the American Association of Feline Practitioners and Academy of  
25 Feline Medicine Advisory Panel on Feline Vaccines *J. Am. Vet. Med. Assoc.* 212, 227-241  
(1998)).

30 In general, it is thought that the risks of side effects to animals is outweighed by the  
benefit imparted by annual vaccine administration even though most vaccines lack supportive  
experimental data documenting a need for annual vaccine administration (1998 Report of the  
American Association of Feline Practitioners and Academy of Feline Medicine Advisory Panel  
on Feline Vaccines *J. Am. Vet. Med. Assoc.* 212, 227-241 (1998)). But today the incidence of  
severe vaccination side effects in animals is more apparent and the risk/benefit ratio for  
annual vaccine administration no longer appears as favorable (Smith *J. Am. Vet. Med. Assoc.*  
207, 421-425 (1995); Hendrick *et al. Cancer Research* 52, 5391-5394 (1992); Duval *et al.*  
35 *Journal of Veterinary Internal Medicine* 10, 290-295 (1996)). Adverse responses range from  
minor local reactions and stinging to severe and sometimes fatal anaphylaxis or development  
of neoplasia. In short, an animal does not benefit and may be placed at serious risk when

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vaccinated unnecessarily (W. Jean Dodds In *Advances in Veterinary Medicine*, Vol. 41, *Veterinary Vaccines and Diagnostics*, Ronald D. Schultz ed. p. 718-719 (1999)). Nevertheless, the companion animal community has – since sufficient information on duration of immunity is lacking – reconciled the risk of over-vaccination with the benefit provided by vaccines in preventing serious disease in animals. But this argument becomes less forceful in light of newer vaccines for diseases that either don't occur with high frequency in unvaccinated animals or that are that are less serious (e.g., Lyme disease, feline leukemia, feline infectious peritonitis, and ringworm).

Conventionally, duration of immunity has been established by selecting an appropriate number of immunologically naive animals (e.g., 20-100), vaccinating with a minimum immunizing dose of the experimental vaccine or placebo, holding the animals in strict isolation for the desired period, and then challenging with the virulent organism. The clinical disease observed in vaccinates is compared to that of non-vaccinates. A disadvantage of this approach is that it is not "real world". That is, the challenge is typically made neither with currently circulating strains of the infectious agent in question nor by the route or means inherent in the field setting. Furthermore, these studies, out of necessity, are long, labor intensive, and costly, as well as often destructive to the animals. It should also be recognized that the longer the study period, the greater the risk that the study animals may inadvertently be exposed to the pathogen that is the subject of the study, thereby invalidating the study. These factors are major considerations when pharmaceutical companies set up duration of immunity studies.

In general, the response of an animal's immune system to infection or immunization can be divided into two functional categories: humoral immunity and cellular immunity. Humoral immunity involves plasma cells (activated B cells) and memory B cells. During a humoral immune response, antigen specific antibodies generated by plasma cells neutralize or opsonize extracellular infectious agents and/or virulence factors. Humoral immunity is important for protection against extracellular phases of systemic viral and bacterial infection.

Cellular immunity involves the production of effector T-cells, memory T-cells (CD4<sup>+</sup>T and CD8<sup>+</sup>T) and NK cells that are responsible for the direct (cell-to-cell) or indirect (via cytokines) destruction of infected cells (Wood PR *et al. Veterinary Immunology & Immunopathology* 54:33-44 (1996) and Allen JE *et al. Immunology Today* 18:387-392 (1997)). Cellular immunity is important in fighting intracellular pathogens.

In addition to humoral and cellular immunity, the non-specific immune system (i.e., phagocytic cells) aids in initial control of infection but is not affected by vaccination or prior exposure.

The immune system self-regulates between a humoral and cellular immune response so that following infection or immunization, one predominates. The type of response that

predominates is related to the type of infectious agent, the route of infection, the pathogenesis of the agent (local versus systemic), and in the case of inactivated vaccines, the inactivating agent and type of adjuvant used.

5 It is evident that some plasma cells must persist for long periods of time, since antibody titers can persist in the blood for years after infection or vaccination without re-exposure. The presence of these pre-existing systemic neutralizing or opsonizing antibodies at the site of infection is effective in preventing infection and providing protective immunity for many years (Plotkin SA & Mortimer EA. Eds., 1994. *Vaccines*. Second Edition, WE Saunders, Philadelphia).

10 Assay-based methods (rather than pathogenic challenge with the virulent infectious agent) may be used as an indication of the presence of adequate immune memory or protective immunity (Olson *et al. Am. J. Vet. Res.* 49, 1460-1466 (1988), Sprent *et al. Science* 265, 1395-1400 (1994), Alderink *et al. Am. J. Vet. Res.* 207, 1016-1018 (1995) Schultz (1995), Smith *Am. J. Vet. Res.* 207, 421-425 (1995)). For many diseases, an adequate  
15 immune memory can be verified by detecting a rapid immune system response (*i.e.*, an anamnestic response) induced by exposure to a specific antigen, for example, detection of antibodies in serum or serologic quantification of antibody formed in response to vaccine administration (Alderink *et al. J. Am. Vet. Med. Assoc.* 207, 1016-1018 (1995); Dodds *Proc. Am. Holistic Vet. Med. Assoc.* 74-80 (1995); Dodds *Vaccine Related issues In Complementary and Alternative Veterinary Medicine*, A.M. Schoen and S.G. Wynn, eds. pp. 701-702 (1997); Schultz (1995); Scott *et al. Feline Pract.* 25, 12-19 (1997)).  
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But immunity against infectious agents for which a cellular response is the primary mode of protection may not correlate with antibody levels. In these cases, an adequate immune memory may be determined by a cellular immunoassay. For example, with feline  
25 rhinotracheitis virus (Johnson *et al. Journal of the American Veterinary Medical Association* 186, 149-152 (1985)) and feline infectious peritonitis virus (Pedersen *Feline Practice* 23, 7-20 (1995)), which are counteracted in companion animals primarily by a cellular immune response, there is no correlation of antibody level with protection. A higher correlation exists with antibody levels for protection against feline leukemia virus (Haffer *et al. Vaccine* 8,12-16  
30 (1990)), where both cellular and humoral immunity are involved. And a strong correlation exists where humoral immunity alone is required for protection, for example, against *Leptospira canicola* (Hartman *et al. Veterinary Immunology and Immunopathology* 7, 245-254 (1984)).

Other factors should be considered when choosing an assay-based procedure to  
35 determine an immunological memory. For example, with diseases that replicate and cause damage primarily on mucosal surfaces – *e.g.*, *Bordetella bronchiseptica*, canine coronavirus, canine parainfluenza, and *Chlamydia psittaci* – serological data may not correlate extensively

with protection (Tennant *et al. Research in Veterinary Science* 51, 11-18 (1991); Bey *et al. American Journal of Veterinary Research* 42, 1130-1132 (1981); and Mitzel *et al. American Journal of Veterinary Research* 38, 1361-1363 (1977)). This is because most of the antibody classes responsible for systemic humoral immunity, as well as the white blood cells responsible for cell mediated immunity, are not present on the mucosal surface and a blood serum based immune response is generally not rapid enough to provide protective antibody or white blood cell levels to the mucosal layer. A serum based immune response will, however, in most cases lessen the severity of mucosally based diseases.

While serological data are useful to provide evidence of an immunological memory against a particular pathogen, quantitative evaluation is generally not informative because the assay methodologies and titer levels have not been standardized between assay methods or laboratories (Luff *et al. Vet. Rec.* 120, 270-273 (1987)). Not only will the use of different assays cause variance, but, even when the same assays are used, the numerical values will depend on the technician's skill, technique, and equipment.

In conclusion, while methods exist to determine whether an animal has a marker of immunity, no practical methodology exists to determine the duration of immunity for animal vaccines. This objective is now met by the method of the present invention as described below.

#### Summary of the Invention

The present invention provides efficient methodology to measure the duration of adequate immune memory for animal vaccines. The invention involves a retrospective analysis utilizing veterinary vaccine administration histories and clinical histories, and markers of immunity of animals in the field (rather than in the laboratory), to derive a vaccine's duration of adequate immune memory. The vaccine may be any vaccine known or to be developed and may be a single component vaccine or may also comprise more than one immunogenic component.

According to the current invention, since challenge occurs in the field under natural conditions, environmental (temperature, humidity, indoor/outdoor), physiological (nutrition, exercise) and psychological (stress of lifestyle or warmth of family setting) variables inherent in the field setting are incorporated in vaccine assessment. This is in contrast to traditional challenge studies using a single laboratory strain of the infectious agent, which may or may not be current with respect to the field epidemiological situation. Thus, in the traditional hold and challenge in confinement approach, these variables are less likely to be representative of the real-world situation and hence, the laboratory measured duration of immunity may not correspond with a field duration of immunity.

In one embodiment the invention concerns a method of determining a duration of adequate immune memory induced by a vaccine for a disease in an animal, the method comprising:

- 5 (a) selecting a plurality of study animals from one or more clinics, where each animal has been vaccinated with the vaccine and where a time since a last vaccination date is at least about one year and the animal has been living in a field environment for at least about one year after the last vaccination date and each animal has a vaccine administration record;
- (b) assigning each animal an indicator of immune memory, such that each animal that does not have a marker of immunity is assigned a first indicator and each animal  
10 that has the marker of immunity is assigned a second indicator; and
- (c) determining the duration of adequate immune memory from: (i) the first indicator and the second indicator, and (ii) the vaccine administration record.

In an embodiment, the method further comprises: (a) assigning each animal that has displayed clinical signs of the disease since the last vaccination or that neither displays an  
15 adequate antibody titer, nor an adequate cellular titer nor an adequate anamnestic response the first indicator; (b) designating each animal which is not assigned the first indicator as either a high risk animal or a low risk animal; (c) assigning each low risk animal that displays either a cellular immune response or that displays either the adequate antibody titer or the sufficient anamnestic response the second indicator; (d) assigning each high risk animal that  
20 has no history of the disease in question and where there is evidence of prevalence of the disease in question in the region the second indicator; and (e) assigning each high risk animal that has no history of the disease in question that displays either the cellular immune response, or the adequate antibody titer or the sufficient anamnestic response, the second indicator.

25 Preferably, the duration of adequate immune memory is determined from a duration of adequate immune memory estimation equation, said duration of adequate immune memory estimation equation derived by a logistic regression analysis of the first and the second indicators and the vaccine administration record. The method of the invention is well-suited for companion animals, especially dogs and cats.

30 Preferably, the step of assigning each animal the indicator of immune memory comprises:

- (a) evaluating a blood serum sample from each animal that has not shown clinical signs of the disease since the last vaccination date to detect an adequate antibody titer of at least about 2 for the disease;
- 35 (b) administering a booster dose of the vaccine to each animal that does not display the adequate antibody titer;

(c) evaluating a blood serum sample from each animal that has received the booster dose 3 days to 28 days following the booster dose to detect an adequate anamnestic response of at least about a 4-fold increase in serum antibody titer; and

(d) assigning the first indicator to each animal that displayed clinical signs of the disease since the last vaccination date or that neither displays the adequate antibody titer nor displays the adequate anamnestic response and assigning the second indicator to each animal that displays either the adequate antibody titer or the sufficient anamnestic response. Another embodiment, of assigning the indicator of immune memory comprises:

(a) evaluating a blood serum sample from each animal that has not shown clinical signs of the disease since the last vaccination date to detect a cellular immune response for the disease;

(b) assigning each animal that has displayed clinical signs of the disease since the last vaccination date or that does not display the cellular immune response the first indicator and assigning each animal that displays the cellular immune response the second indicator.

In still another preferred embodiment, the step of determining the duration of adequate immune memory from the first and the second indicators and the vaccine administration record comprises:

(a) determining an enrollment date for each animal, the enrollment date being when evaluation of the animal to detect the marker of immunity was begun;

(b) assigning a start of study date as the enrollment date for a first animal of the plurality of animals enrolled in the study;

(c) assigning a variable  $X_j$  for each animal as a number of days between the start of study date and the enrollment date for the animal;

(d) assigning a variable  $X_i$  for each animal as a number of days between the last vaccination date for the animal and the enrollment date for the animal.

(e) determining the duration of adequate immune memory from a duration of adequate immune memory estimation equation, said duration of adequate immune memory estimation equation derived by a logistic regression analysis of the first and second indicators and the variables  $X_j$  and  $X_i$ .

In an embodiment, the duration of adequate immune memory estimation equation is in a form  $\text{logit}(E) = \beta_0 + \beta_1 X_{Di} + \beta_2 X_{Cv} + C_k$ , where:

$E$  is a desired level of efficacy;  
 $X_{Di}$  is the duration of adequate immune memory;  
 $X_{Cv}$  is a mean of  $X_j$ ;  
 $C_k$  is a constant representing a random effect to account for variation between the clinics and is derived by logistic regression; and

$\beta_0, \beta_1, \beta_2$  are constants derived by logistic regression.

In a more preferred embodiment thereof, the model for the logistic regression to derive the values of  $C_k, \beta_0, \beta_1$ , and  $\beta_2$  is in a form  $\text{logit}(p) = \beta_0 + \beta_1 X_i + \beta_2 X_j + C_k$ , where:

$\text{logit}(p)$  is a vector representing the immune statuses for the animals; and

5  $C_k$  is the clinic from which each animal was selected.

These and other features, aspects, and advantages of the invention will become better understood with reference to the following detailed description, examples, and appended claims.

10 Detailed Description of the Invention

As used herein, the term "duration of adequate immune memory" for a particular vaccine means the estimated time that an animal, vaccinated with the vaccine, will display a marker of immunity to the disease for which the vaccine is prescribed.

As used herein, the term "marker of immunity" means any indication that an animal-  
15 which has not shown clinical signs for the disease in question since the last vaccination date-  
has an immunologic memory to the disease. According to the definition of "marker of  
immunity", an animal that has shown clinical signs of the disease in question since the last  
vaccination date does not have a marker of immunity for the disease. Examples of markers  
of immunity include those detected by serologic assays, such as circulating antibody titers,  
20 secretory antibody titers, cellular responses (e.g., B or T-cell activity), cytokine levels (e.g.,  
tumor necrosis factor, gamma interferon, etc.) and other assays known to those skilled in the  
art. Other markers are detected by non-serologic analysis, for example, if, in high exposure  
risk area (e.g., high disease prevalence or high population density), the animal does not  
display the disease's symptoms. This is particularly useful when the disease symptoms are  
25 unique. Suitable markers of immunity vary widely with the disease and the animal's exposure  
risk. One skilled can readily choose the appropriate marker of immunity depending on the  
disease, the animal, and the risk factors.

As used herein, the term "animal" means any animal that can benefit by vaccination,  
including, but not limited to, companion animals; livestock and farm animals, such as cows,  
30 sheep, goats, and swine; fowl, such as chickens, turkeys, and ducks, and wildlife such as  
deer and fish. Preferably, the animal is a companion animal.

As used herein the term "companion animal" is any tamed or domesticated animal  
that is kept for human companionship, for example, cats, dogs, horses, birds, snakes, ferrets,  
hamsters, and the like.

35 As used herein, a "field environment" means a non-laboratory setting where the  
animal lives under the typical conditions and setting for which the animal is adapted or bred.

For example, the field environment for a companion animal is the typical conditions and setting of the pet/owner relationship, such as the owner's household, yard, or barn.

As used herein, the term "clinic" means a particular sampling pool of animals. For example, a clinic for a particular study may be defined as a particular geographical area. One  
5 of skill in the art will readily know how to define the clinic for a duration of adequate immune memory study depending on the specific disease and animal under evaluation. Examples of clinics include but are not limited to veterinary hospitals, feed lots, farms, and barns. The number, scope, and selection of clinics for a particular study will vary with the nature and prevalence of the disease and one skilled in the art will know how to select such variables to  
10 ensure validity of the study.

The invention is of a general nature and useful to establish the duration of adequate immune memory induced by any vaccine, known or to be discovered, in any animal. For example, suitable animals include but are not limited to, farm animals such as, bovine, equine, swine, poultry, as well as wildlife, such as fish and birds. The invention is particularly  
15 suited to measure a vaccine's duration of adequate immune memory in companion animals such as dog or cats. The vaccine type may be any of those known in the art or to be developed, for example, modified live vaccines, killed vaccines, DNA vaccines, vectored vaccines, or subunit vaccines or any other vaccine type known or to be discovered. Examples of cat and dog vaccines include both core and non-core vaccines. Examples of  
20 feline core vaccines include vaccines effective for panleukopenia, feline viral rhinotracheitis, calicivirus and rabies, while feline non-core vaccines include vaccines for infectious peritonitis, leukemia, and *Chlamydia psittaci*. Canine core vaccines include vaccines designed for distemper, parvovirus, adenovirus, and rabies, while canine non-core vaccines include vaccines against parainfluenza, Bordetella, Borrellia, and Leptospira.

25 The vaccine administration method may be any method known in the art such as, parenteral or intranasal.

In one embodiment, the invention provides a method of determining a duration of adequate immune memory induced by a vaccine for a disease in an animal, the method comprising:

- 30 (a) selecting a plurality of study animals from one or more clinics, where each animal has been vaccinated with the vaccine and where a time since a last vaccination date is at least about one year and the animal has been living in a field environment for at least about one year after the last vaccination date and each animal has a vaccine administration record;
- (b) assigning each animal an indicator of immune memory, such that each  
35 animal that does not have a marker of immunity is assigned a first indicator and each animal that has the marker of immunity is assigned a second indicator; and



(c) determining the duration of adequate immune memory from: (i) the first indicator and the second indicator, and (ii) the vaccine administration record.

The number of animals employed in the study should be statistically significant. One skilled in art can readily determine a statically significant group of animals depending on the area's disease prevalence, population density, and the disease under study, using well known techniques (Steel, R.G.D. and Torrie, J.H. (1980), *Principles and Procedures of Statistics: A Biometrical Approach*. 2nd Edition; and Cannon, R. M. and Roe, R.T. (1982) *Livestock Disease Surveys: A Field Manual for Veterinarians*, both of which are incorporated herein by reference).

Preferably, at least about 100 animals are included in the study, more preferably about 200 to about 10,000 animals, most preferably about 300 to about 1000 animals. In general, the larger the number the more accurate the study, although the difficulty in selecting the animals and collecting the data rises proportionally.

Preferably, each study animal was vaccinated with the same vaccine (*i.e.*, prepared by the same manufacturer by the same method), by the same vaccine administration route (*e.g.*, injection, subcutaneous, parenteral, intramuscular, intranasal), at the same vaccination site. Preferably, animals to which the vaccine was improperly administered (*e.g.*, the instructions on the label were not followed or an expired vaccine was administered, *etc.*) are excluded from the study group.

The plurality of study animals that comprise the study group may be selected from clinics of different risk and geographical locations. One skilled in the art will readily be able to choose the study group according to the vaccine, disease, animal, marker of immunity to be evaluated, and the animal's exposure risk. When, because of the nature of the study, the animals are selected from a high exposure risk area, the preferred marker of immunity is lack of clinical signs for the disease. Preferably, such disease signs would be pathognomonic. On the other hand, when the animals are selected from a low exposure risk area, the preferred marker of immunity is evidence of a vaccine response (*e.g.*, serologic assay). These markers are preferred because when the study animals are selected from a high risk area, actual disease exposure may be the origin of a serologic immune memory rather than the vaccine, thus serologic data are inconclusive. And, of course, when the study animals are selected from a low exposure risk area, the absence of clinical signs is not a useful marker of immunity.

Factors associated with disease exposure risk include the animal's environment (*inter alia*, the prevalence of the disease and animal population density in the animal's particular geographic location, the number of like animals in the household (when the animal is a companion animal), whether the animal is an indoor or outdoor animal, whether the animal is periodically housed in boarding houses or kennels, and if the animal has occasion to roam

free), age, general health, current medications, sex, and any diseases that the animal has contracted.

An animal's exposure risk to a specific disease in a particular geographical area is readily determined by one skilled in the art. Risk factors may be estimated by various means.

- 5 Such methods of disease risk estimation may include published data, epidemiological modeling using published factors, regional/local Diagnostic Laboratory sample results (i.e., most labs keep records of submissions and diagnosis, enabling a calculation of % positive) and clinic surveys.

- 10 In a preferred embodiment of the invention, the minimum age for animals is 6 to 8 weeks. This is because high levels of maternal antibodies in young animals (under about 6 to 8 weeks of age) can prevent some vaccines from being effective. An animal's immune system responds most effectively to vaccination after decay or disappearance of the maternal antibodies (Schultz *Vet. Med.* 233-254, March 1998 and Serge Martinod In *Advances in Veterinary Medicine*, Vol. 41, *Veterinary Vaccines and Diagnostics*, Ronald D. Schultz ed. p. 15 661 (1999)).

- 20 The animal's vaccine administration and clinical history are obtained to determine the last vaccination date, whether the animal has been living in a field environment for about a year after the last vaccination date, and whether the animal has manifested clinical signs of the disease since the last vaccination date. Preferably the animal's vaccine administration and clinical history also includes the vaccine's route of administration and administration site, manufacturer, lot and serial number, and expiration date. Even more preferably, the animal's history further includes factors that indicate the animal's risk of contracting the disease, for example, the animal's environment as well as the animal's age, general health, current medications, sex, breed, whether the animal has been neutered or spayed, and any diseases 25 that the animal has contracted. Data concerning the animal's environment preferably includes such factors as the prevalence of the disease in the animal's particular geographic location, population density of the animal under study, the number and type of like animals in the household, whether the animal is an indoor or outdoor animal, whether the animal is periodically housed in boarding houses or kennels, and whether the animal has occasion to 30 roam free. Of course, the higher the animal's incidence of contact with a large number of other like animals, the higher the animal's exposure risk. Poor general health, old age, or other conditions that compromise the immune system will also put an animal at a higher risk of contracting the disease in question. Furthermore, past incidence of disease and current prescription medications may also put the animal at a higher risk (1998 Report of the 35 American Association of Feline Practitioners and Academy of Feline Medicine Advisory Panel on Feline Vaccines *J. Am. Vet. Med. Assoc.* 212, 227-241 (1998)).

The animal's vaccine administration and clinical history are available from veterinary and animal owner records. Currently no prescribed system of record keeping exists among veterinarians, but it is recommended by the companion animal community that record keeping be meticulous and methodical (Report of the American Association of Feline Practitioners and Academy of Feline Medicine Advisory Panel on Feline Vaccines *J. Am. Vet. Med. Assoc.* 212, 227-241 (1998)). One method to facilitate transfer of veterinary and companion animal owner records to the manufacturer conducting a duration of adequate immune memory study is by supplying a prestamped questionnaire to the veterinarian for compiling the animal's clinical and vaccine administration history. The questionnaire may have a preferred time of submission, after which, the companion animal owner and the veterinarian may complete and submit it to the manufacturer. Peel off vaccine labels, to be affixed to the questionnaire can facilitate such record keeping.

Once the study group has been selected and the clinical and vaccine administration histories examined and compiled, the immune status for each study animal is assigned. A first indicator is assigned to each animal that does not display a marker of immunity, preferably the first indicator is a 0. And a second indicator is assigned to each animal that displays a marker of immunity, preferably the second indicator is a 1.

The particular marker of immunity assayed for will depend upon the particular disease for which the vaccine is being tested. For the purposes of the present invention, each animal that has manifested clinical signs of the disease under study, since the last vaccination date does not have a "marker of immunity". Markers of immunity and methods for their assay are well known in the art, and the ordinary artisan will know which procedure to select depending on the study. For example, a blood serum sample, obtained from the animal's owner or veterinarian, may be analyzed for the presence of circulating antibodies. Another marker of immunity is an anamnestic response to the disease in question i(*Manual of Clinical Laboratory Immunology*, Fifth Edition, Editor: Noel R. Rose *et al.* (1997); *Manual of Clinical Microbiology*, 7th Edition, Editor: Patrick R. Murray *et al.* (1999); *Clinical Microbiology Procedures Handbook*, Editor: Henry D. Isenberg *et al.* (1992); S.Faine, *Leptospira and Leptospirosis*, Second Edition, (1994)).

Where the primary immune response for a specific disease is cell-mediated, there are a variety of assays that can be used to identify animals with a marker of immunity. For example see, (*Manual of Clinical Laboratory Immunology*, Fifth Edition, Editor: Noel R. Rose *et al.* (1997)). Table 1 below list a few examples of suitable markers of immunity for a variety of vaccines.

TABLE 1: Markers of immunity for various canine vaccines

Disease for which the vaccine is prescribed	Marker	References
Rabies	Seroneutralizing titers	World Health Organization. 1992. <i>Expert Committee on Rabies, 8th Report</i> . World Health Organization, Technical Report Series no. 824, Geneva.
Canine Distemper	Seroneutralizing titers Ab	Cooper et al. <i>Bulletin Mensuel de la Societe Veterinaire Pratique de France</i> 75, 13 1-152 (1991). Auby et al. <i>Recueil de Médecine Vétérinaire</i> 150, 33-36 (1974).
Canine Parvovirus	Seroneutralizing titers Ab	Pollock et al. <i>Journal of the American Veterinary Medical Association</i> 180, 37-43 (1979).
Canine Adenovirus CAV-1	Seroneutralizing titers Ab	Hartman et al. <i>Veterinary Immunology and Immunopathology</i> 7, 245-254 (1984).
<i>Leptospira canicola</i> and <i>icterohaemorrhagiae</i>	Neutralizing IgG titers	Hartman et al. <i>Veterinary Immunology and Immunopathology</i> 7, 245-254 (1984).
Canine Coronavirus	Secretory IgA	Tennant et al. <i>Research in Veterinary Science</i> 51, 11-18 (1991).
Bordetella Bronchiseptica/Canine Parainfluenza	Secretory IgA	Bemis DA. 1976. <i>Bordetella bronchiseptica</i> in dogs: Bacteriological studies, pathogenesis, immune response and antibiotic treatment. Ph.D. Thesis. Cornell University
Panleukopenia	Seroneutralizing titers Ab	Scott et al. <i>Journal of the American Veterinary Medical Association</i> 156, 439-453 (1970). Johnson et al. <i>Journal of the American Veterinary Medical Association</i> 158, 876-884 (1971).
Rhinotracheitis	Role of cell mediated immunity	Johnson et al. <i>Journal of the American Veterinary Medical Association</i> 186, 149-152 (1985).
Rabies	Seroneutralizing titers	World Health Organization. 1992. <i>Expert Committee on Rabies, 8th Report</i> . World Health Organization, Technical Report Series no. 824, Geneva.
Calicivirus	Seroneutralizing titers Ab	Johnson et al. <i>Journal of the American Veterinary Medical Association</i> 186, 149-152 (1985).
<i>Chlamydia psittaci</i>	Secretory IgA	Mitzel et al. <i>American Journal of Veterinary Research</i> 38, 1361-1363 (1977).
FeLV	Seroneutralizing titers Ab	Haffer et al. <i>Vaccine</i> 8:12-16 (1990). Tompkins et al. <i>Companion Animal Practice</i> July: 15-26 (1988).
Infectious Peritonitis	Salivary IgA	Pedersen <i>Feline Practice</i> 23:7-20 (1995).

In a preferred embodiment of the invention, a marker of immunity is established when the animal's blood serum contains either a blood serum antibody titer level of at least about 2 or if the animal displays a strong anamnestic response upon administration of a booster dose of the vaccine. A strong anamnestic response is a 4-fold increase in serum antibody titer when measured 3 days to 28 days following administration of a booster dose of the vaccine in question. The conventional practice is to draw a blood serum sample then administer the booster dose. Then, within 3 days to 28 days, a second blood serum sample is taken. Sera from the first and second blood samples are tested for antibody in parallel. In the presence of

immunological memory in a healthy individual, antibody levels in the second serum sample will be 4-fold greater than levels in the first sample.

Once the immune status for each animal is established as described above, the data for the study group will be compiled and analyzed to determine the duration of adequate immune memory. Each animal's clinic ( $C_k$ ), enrollment date, and last vaccination date will be recorded, and a value in days will be determined for each of  $X_i$  and  $X_j$ , where:

$X_i$  is the  $i^{\text{th}}$  animal's time in days between the animal's last vaccination date for the disease and the animal's enrollment date;

$X_j$  is the  $j^{\text{th}}$  animal's time in days between the animal's enrollment date and the start of study date.

As used herein, the "enrollment date" for each animal is the day on which the animal was evaluated for a marker of immunity. If the animal is assayed for more than one marker of immunity, the enrollment date is the date on which the animal was evaluated for the first marker of immunity. For example, when the marker of immunity is detection of antibody titer levels, the enrollment date is the day on which the first blood sample is drawn for antibody titer analysis. If the antibody titer analysis is negative, and the animal is evaluated for an alternate marker of immunity, such as an anamnestic response, the enrollment date is still the day on which the first blood sample is drawn for antibody titer analysis.

Herein, when referring to the "last vaccination date", the "vaccination date" means the day on which the prescribed vaccine administration protocol is concluded. Note that this date will not necessarily correspond to the date a particular vaccine dose is administered. Often, companion animal vaccination involves a series of vaccine administrations at short time intervals to establish immunity. For example, it may be necessary to give two or more doses of a killed vaccine within a short time period to attain immunity, whereas, in the same animal, modified live vaccines often immunize after only one administration (Report of the American Association of Feline Practitioners and Academy of Feline Medicine Advisory Panel on Feline Vaccines *J. Am. Vet. Med. Assoc.* 212, 227-241 (1998)). Another example where vaccination date must be differentiated from vaccine administration date is where young animals (less than about 8 to 16 weeks) are administered multiple vaccine doses, spaced at 2 to 6 week intervals. This vaccine administration protocol is prescribed because maternally inherited antibodies may be present in the animals blood serum. Maternally inherited antibodies can negate vaccination and since it is not known exactly when these antibodies will dissipate, multiple vaccine administrations are recommended. For most companion animals, maternally inherited antibodies disappear after about 16 weeks of age. For example, the recommended vaccine administration schedule for canine distemper in puppies is a series of three parenteral injections of a modified live vaccine (*i.e.*, at 6 to 8 weeks, at 10 to 12 weeks, and at 14-16 weeks) (Council Report *Journal of the American Veterinary Medical Association* 195,

314-317 (1989)). Accordingly, in this case, the vaccination date is the date on which the 14-16 week vaccine injection is administered. In the current invention, in cases where the prescribed vaccine administration protocol is not followed for a particular animal, it is preferable that the animal be omitted from the study.

5 In one embodiment of the invention, the vaccine's duration of adequate immune memory is estimated by a duration of adequate immune memory prediction equation, appropriate for the statistical model. The prediction equation may be derived by logistic regression analysis of the immunity data obtained from the animals enrolled in the study. Derivation of prediction equations by logistic regression analysis of raw data is well known to  
10 those of skill in the art. Exemplary models can be found in the following references: Agresti (1990), *Categorical Data Analysis*, 84-91; Beitler *et al. Biometrics*, 41, 991-1000 (1985); Davidian *et al. Biometrika* 80, 475-488 (1993); Harville *et al. Biometrics* 40, 393-408 (1984); Hosmer *et al. (1989), Applied Logistic Regression*; Longford *Computational Statistics and Data Analysis* 17, 1-15 (1994).

15 Once the prediction equation for a particular vaccine is derived, it may be solved to obtain the duration of adequate immune memory for the vaccine under study by imputing a desired efficacy level. The desired efficacy level is the percentage of vaccinated animals that would not manifest clinical signs of the disease if a statistically significant group was exposed to the disease in question. Or, in other words, the efficacy level is the probability that a  
20 particular animal will show clinical manifestations if exposed to a virulent challenge of the disease. Efficacy is a relative term and no vaccine will induce complete immunity to all animals in the field. To be federally licensed, the vaccinated group must show significantly less disease than the non-vaccinated control group upon a virulent challenge. For most diseases, for USDA approval, the efficacy level must be 80% (*i.e.*, 80% of the vaccinates  
25 must not show illness, Serge Martinod In *Advances in Veterinary Medicine*, Vol. 41, *Veterinary Vaccines and Diagnostics*, Ronald D. Schultz ed. p. 635 (1999)).

The preferred prediction equation is:

$$\text{logit}(E) = \beta_0 + \beta_1 X_{DI} + \beta_2 X_{CV} + C_k$$

where:

30 E is the desired level of efficacy;  
X<sub>DI</sub> is the duration of adequate immune memory;  
X<sub>CV</sub> is the mean X<sub>j</sub> value obtained using a means averaging program such as the MEANS procedure of SAS (this value represents a covariate to adjust for possible seasonal differences);

35 C<sub>k</sub> is a constant representing the random effect in the model to account for the possible variation between clinics, C<sub>k</sub> is derived by logistic regression; and

$\beta_0, \beta_1, \beta_2$  are constants derived by logistic regression.

The preferred statistical model for the logistic regression to derive the values of  $C_k$ ,  $\beta_0$ ,  $\beta_1$ , and  $\beta_2$  is:

$$\text{logit}(p) = \beta_0 + \beta_1 X_i + \beta_2 X_j + C_k$$

where  $X_i$ , and  $X_j$ , are defined above,  $C_k$  is the  $k^{\text{th}}$  clinic (*i.e.*, the clinic corresponding to the particular animal), and  $\text{logit}(p)$  is a vector representing the immune status indicators for each animal in the study group (*e.g.*, a vector consisting of 0s and 1s). Thus  $p$  becomes the probability that a animal in the study will display a marker of immunity.

Any suitable statistical program may be used for the logistic regression. Example of suitable models may be found in the SAS software package (*e.g.*, Version 8), well known to those in the art. The NLMIXED procedure in SAS is preferred logistic regression program. But the LOGISTIC, TLOGISTIC, GENMOD or CATMOD procedures in the SAS package may also be used.

#### Protocols for Determining Markers of Immunity

##### Serological analysis of canine distemper virus

Norden Laboratories Dog Kidney cells of passage range 126-143 are plated at 16,000 cells per plate well in Eagles minimal essential medium/10% Fetal bovine serum. The plates are incubated at 37°C, 5% CO<sub>2</sub> in a humidified environment for 24 hours until the desired confluency of 20-30% is achieved. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in Eagles minimal essential medium. Each test well of the diluted serum is combined with an equal volume of virus suspension containing 100-316 tissue culture infectious dose 50% (TCID<sub>50</sub>) of canine distemper virus. Typically, one can use a non-cytopathic virus. The serum/virus mixture is incubated for an hour to allow neutralization of virus by specific antibody. Challenge virus is titrated to verify the amount of virus used in the assay (back- titration). Following incubation, the fluid contents of the plates are removed and the plates are inoculated with the serum/virus mixture, four replicates per serum dilution. Plates are incubated for 6±1 days at 37°C, 5% CO<sub>2</sub> in a humidified environment. The plates are then fixed with 80% acetone, rinsed and stained with direct fluorescein isothiocyanate labeled anti-CDV polyclonal antibody. Following the staining procedure, the plates are read for the presence or absence of viral specific fluorescence. Wells exhibiting no fluorescence ("FA") are scored as a "+" denoting the presence of neutralizing antibody. Wells containing FA are scored as a "-" denoting the lack of neutralizing antibody. Serum neutralizing titer is calculated by Spearman-Kärber. An assay must fulfill the following criteria to be considered valid: 1) The back-titer must be within 100-316 TCID<sub>50</sub>; 2) The positive control serum must be within the established range (typically, 36-145); 3) The negative control serum must have a titer of ≤2.

Serological analysis of canine adenovirus type 2

Norden Laboratories Dog Kidney cells of passage range 126-143 are plated at 16,000 cells per plate well in Eagles minimal essential medium/10% Fetal bovine serum. The plates are incubated at 37°C, 5% CO<sub>2</sub> in a humidified environment for 3-5 days until the desired confluency of 85-95% is achieved. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in Eagles minimal essential medium. Each test well of the diluted serum is combined with an equal volume of virus suspension containing 100-316 tissue culture infectious dose 50% (TCID<sub>50</sub>) of canine adenovirus type 2. Typically, one can use a non-cytopathic virus. The serum/virus mixture is incubated for an hour to allow neutralization of virus by specific antibody. Challenge virus is titrated to verify the amount of virus used in the assay (back-titration). Following incubation, the fluid contents of the plates are removed and the plates are inoculated with the serum/virus mixture, four replicates per serum dilution. Plates are incubated for 7±1 days at 37°C, 5% CO<sub>2</sub> in a humidified environment. The plates are then fixed with 80% acetone, rinsed and stained with direct fluorescein isothiocyanate labeled anti-CAV polyclonal antibody. Following the staining procedure, the plates are read for the presence or absence of viral specific fluorescence. Wells exhibiting no FA are scored as a "+" denoting the presence of neutralizing antibody. Wells containing FA are scored as a "-" denoting the lack of neutralizing antibody. Serum neutralizing titer is calculated by Spearman-Kärber. An assay must fulfill the following criteria to be considered valid: 1) The back-titer must be within 100-316 TCID<sub>50</sub>; 2) The positive control serum must be within the established range (typically, 25-99); 3) The negative control serum must have a titer of ≤2.

Serological analysis of canine parainfluenza virus

Norden Laboratories Dog Kidney cells of passage range 126-143 are plated at 16,000 cells per plate well in Eagles minimal essential medium/10% Fetal bovine serum. The plates are incubated at 37°C, 5% CO<sub>2</sub> in a humidified environment for 24 hours until the desired confluency of 20-30% is achieved. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in Eagles minimal essential medium. Each test well of the diluted serum is combined with an equal volume of virus suspension containing 100-316 tissue culture infectious dose 50% (TCID<sub>50</sub>) of canine parainfluenza virus. Typically, one can use a non-cytopathic virus. The serum/virus mixture is incubated for an hour to allow neutralization of virus by specific antibody. Challenge virus is titrated to verify the amount of virus used in the assay (back-titration). Following incubation, the fluid contents of the plates are removed and the plates are inoculated with the serum/virus mixture, four replicates per serum dilution. Plates are incubated for 6±1 days at 37°C, 5% CO<sub>2</sub> in a humidified environment. The plates are then fixed with 80% acetone, rinsed and stained with direct fluorescein isothiocyanate labeled anti-



CPI polyclonal antibody. Following the staining procedure, the plates are read for the presence or absence of viral specific fluorescence. Wells exhibiting no FA are scored as a "+" denoting the presence of neutralizing antibody. Wells containing FA are scored as a "-" denoting the lack of neutralizing antibody. Serum neutralizing titer is calculated by Spearman-Karber. An assay must fulfill the following criteria to be considered valid: 1) The backtiter must be within 100-316 TCID<sub>50</sub>; 2) The positive control serum must be within the established range (typically, 32-82); 3) The negative control serum must have a titer of  $\leq 2$ .

#### Serological analysis of parvovirus

Norden Laboratories Dog Kidney cells of passage range 126-143 are plated at 16,000 cells per plate well in Eagles minimal essential medium/10% Fetal bovine serum. The plates are incubated at 37°C, 5% CO<sub>2</sub> in a humidified environment for 24 hours until the desired confluency of 20-30% is achieved. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in Eagles minimal essential medium. Each test well of the diluted serum is combined with an equal volume of virus suspension containing 100-316 tissue culture infectious dose 50% (TCID<sub>50</sub>) of canine parvovirus. Typically, one can use a non-cytopathic virus. The serum/virus mixture is incubated for an hour to allow neutralization of virus by specific antibody. Challenge virus is titrated to verify the amount of virus used in the assay (back-titration). Following incubation, the fluid contents of the plates are removed and the plates are inoculated with the serum/virus mixture, four replicates per serum dilution. Plates are incubated for 3-4 days at 37°C, 5% CO<sub>2</sub> in a humidified environment. The plates are then fixed with 80% acetone, rinsed and stained with direct fluorescein isothiocyanate labeled anti-CPV polyclonal antibody. Following the staining procedure, the plates are read for the presence or absence of viral specific fluorescence. Wells exhibiting no FA are scored as a "+" denoting the presence of neutralizing antibody. Wells containing FA are scored as a "-" denoting the lack of neutralizing antibody. Serum neutralizing titer is calculated by Spearman-Karber. An assay must fulfill the following criteria to be considered valid: 1. The back-titer must be within 100-316 TCID<sub>50</sub>; 2) The positive control serum must be within the established range (typically, 88-352); 3) The negative control serum must have a titer of  $\leq 2$ .

#### Serological analysis of canine coronavirus

Norden Laboratories Feline Kidney cells of passage range 85-92 are plated at 50,000 cells per plate well in Eagles minimal essential medium+10% Fetal bovine serum. The plates are incubated at 37°C, 5% CO<sub>2</sub> in a humidified environment for 3 $\pm$ 1 days until the desired confluency of 100% is achieved. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in BME+10mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)+4 $\mu$ g/mL polybrene. Each test well of the diluted serum is combined with an equal volume of virus suspension containing 100-316

tissue culture infectious dose 50% (TCID<sub>50</sub>) of canine coronavirus. The serum/virus mixture is mixed and incubated for an hour to allow neutralization of virus by specific antibody. Challenge virus is titrated to verify the amount of virus used in the assay (back-titration). Following incubation, the fluid contents of the plates are removed and the plates are inoculated with serum/virus mixture, four replicates per serum dilution. Plates are incubated for 5±1 days at 37°C, 5% CO<sub>2</sub> in a humidified environment. The wells are then read for the presence of virus by cytopathic effect (CPE). Wells exhibiting no CPE are scored as a "+" denoting the presence of neutralizing antibody. Wells containing CPE are scored as a "-" denoting the lack of neutralizing antibody. Serum neutralizing titer is calculated by Spearman-Kärber. An assay must fulfill the following criteria to be considered valid: 1) The back-titer must be within 100-316 TCID<sub>50</sub>; 2) The positive control serum must be within the established range (typically, 27-108); 3) The negative control serum must have a titer of ≤2.

#### Serological analysis of *Leptospira canicola*

Living late logarithmic or early stationary phase cultures of *Leptospira canicola* are use for the assay. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in diluent. An equal volume of culture is added to the diluted serum. The serum/bacterial mixture is mixed and incubated for 2 to 4 hour to allow agglutination of bacteria by specific antibody. The suspension in each well is examined microscopically for agglutination. The endpoint is the highest well in which 50% agglutination is recorded, measured by comparison of the density of unagglutinated leptospire with a control suspension. An assay must fulfill the following criteria to be considered valid: 1) The number of bacteria must used to challenge each well must be between 2 x 10<sup>8</sup> and 1 x 10<sup>9</sup>/mL; 2) The positive control serum must be within the established range; 3) The negative control serum must have a titer of ≤2.

#### Serological analysis of *Leptospira icterohaemorrhagiae*

Living late logarithmic or early stationary phase cultures of *Leptospira icterohaemorrhagiae* are use for the assay. Heat inactivated canine serum samples, positive control and negative control sera are serially diluted 2-fold to appropriate levels in diluent. An equal volume of culture is added to the diluted serum. The serum/bacterial mixture is mixed and incubated for 2 to 4 hour to allow agglutination of bacteria by specific antibody. The suspension in each well is examined microscopically for agglutination. The endpoint is the highest well in which 50% agglutination is recorded, measured by comparison of the density of unagglutinated leptospire with a control suspension. An assay must fulfill the following criteria to be considered valid: 1) The number of bacteria must used to challenge each well must be between 2 x 10<sup>8</sup> and 1 x 10<sup>9</sup>/mL; 2) The positive control serum must be within the established range; 3) The negative control serum must have a titer of ≤2.

### Examples

The following examples further illustrate the methods of the present invention. It is to be understood that the present invention is not limited to the specific details of the examples provided below.

5        EXAMPLE 1: A retrospective study to determine the duration of adequate immune memory of the various viral and bacterial components of Vanguard® 5/L or Vanguard Plus® 5/L vaccines in dogs by measuring antibody titers and antibody response to revaccination.

1.        VACCINE

10        Generic Name: Canine distemper, adenovirus type 2, parvovirus, parainfluenza vaccine, *modified* live virus vaccine (MLV)  
*Leptospira canicola-icterohaemorrhagiae* bacterin  
(inactivated whole cultures)

Trade Name: Vanguard® Plus 5/L (APHIS Product Code 4639.22).  
US Vet. License No.: 189

15        Formulation: Commercial vaccine

2.        STUDY ANIMALS:

Species                      Canine                      Type: Mixed and purebred dogs  
Breed/Strain: Any                      Sex: M & F (intact or neutered)  
Description: Adults  
20        Origin: Privately owned veterinary patients  
Initial Age:                      Min: 2                      Max: None                      Units: Years  
Weights:                      Min: None                      Max: None                      Units: Pounds

3.        MANAGEMENT:

a.        Site Specifications

25        This study is a controlled clinical study conducted at multiple sites over diverse geographic regions in the United States and Canada. One protocol common to all sites is used. Approximately 40-60 veterinary practices will participate in the study. Each individual study site is expected to enroll a minimum of 5 dogs.

30        The sites are small animal veterinary practices whose clientele, record keeping and compliance will provide a large proportion of enrolled patients. The practices should have at least one veterinarian (the Study Director or Examining Veterinarian designated by the Study Director) who is experienced in general veterinary practice and medicine. The practices will have a history of using Vanguard® 5/L or Vanguard Plus® 5/L over the last five years. The owners of the dogs enrolled in the study shall be willing to participate in the study until  
35        completion of all observations and collection of all samples.

b. Pretreatment

Medical management will follow normal accepted clinical practice for each study animal. Treatments listed under "Exclusion Criteria" will not be administered and the use of other concomitant medications are recorded.

5 c. Identification Method

Individual animal identification as provided by owners and unique case report numbers.

d. Housing

10 Dogs are kept in normal domestic environments and can be housed either in the client's house or in separate animal accommodation. Dogs can be housed with or without other animals.

e. Feeding and Watering Methods

Normal practice.

4. STUDY DESIGN:

No.	Treatment Description	Time Since Last Vaccination	Route	Animals/ Treatment
T1.	Vanguard Plus® 5/L	≥12 months	Subcutaneous	Approx. 500-1000

15

5. PROCEDURES:

20 Dogs are selected for the study from animals presented to each site. The medical records of each site are reviewed to identify adult dogs that have received a priming vaccination series, but have not been vaccinated for 12 months or longer. The most recently administered vaccine is Vanguard® 5/L or Vanguard® Plus 5/L. An attempt is made to identify a range of animals that have not been vaccinated in 12 to 48 months (or longer), such that immune status can be assessed over an extended period since last vaccination. Duration of adequate immune memory to the individual Vanguard® vaccine components are determined in the selected dogs based on serum antibody titers and/or an anamnestic response to revaccination with Vanguard® Plus 5/L.

25

General information for all identified candidate cases are recorded and eligibility assessed. Only animals that satisfy all of the inclusion criteria and for which none of the exclusion criteria apply are selected for enrollment in the study.

30 Healthy dogs, 2 years of age or older, which have not been vaccinated in the past 12 months, or longer, are eligible for enrollment. Owners of these animals are contacted by telephone and asked to participate in the study. Reasons for non-participation in the study are recorded.

a. Inclusion Criteria

1. Any breed, sex or weight of dog.

2. Healthy dogs, 2 years of age or older.
  3. Dog must not have received a mono- or polyvalent vaccine containing canine distemper adenovirus type 2, parvovirus, parainfluenza vaccine, modified live virus vaccine; *Leptospira canicola-icterohaemorrhagiae* bacterin within the past 12 months, or longer. (Dogs may have received rabies, *Bordetella*, *Borrelia*, or *Giardia* vaccinations).
  4. Dog must have a documented history of vaccination with at least two doses of a polyvalent vaccine (containing canine distemper, adenovirus type 2, parvovirus, parainfluenza vaccine, modified live virus vaccine; *Leptospira canicola-icterohaemorrhagiae* bacterin, from any manufacturer) as a priming dose (puppy or initial vaccination series), with the last dose given when the animal was 12 weeks of age or older.
  5. Dog must have received at least one additional polyvalent vaccine 9-14 months after the initial priming series.
  6. The most recent vaccine administered must be Vanguard® 5/L or Vanguard Plus® 5/L.
  7. The owner/authorized agent must agree to return the study animal to the veterinary practice for evaluation at the required times.
  8. The owner/authorized agent must give written informed consent for the animal to participate in the study.
  9. The owner must complete the animal lifestyle and disease risk assessment questionnaire.
- b. Exclusion Criteria
1. Dogs with a history of intolerance/allergy to vaccinations.
  2. Pregnant animals.
  3. Dogs with severe systemic disease, whether or not it is a constant threat to life (renal failure, hepatic failure, terminal neoplasia).
  4. Dogs treated with any immunosuppressive drugs (corticosteroids including prednisone, and dexamethasone, or other immunosuppressives such as cyclophosphamide, azathioprine, and gold sodium thiomalate, etc.) within the past 10 days.
  5. Dogs treated with repository anti-inflammatory drugs (e.g., methyl-prednisolone acetate) within the last 30 days.
- c. Pre-Vaccination Examination. Blood Collection and Processing
- On the enrollment date all dogs will receive a physical examination to determine the dog's general health. The owner will complete a questionnaire to document the lifestyle and risk of disease exposure of their dog.
- On the enrollment date (once the dog is confirmed to meet inclusion criteria), approximately ten (10) mL of blood is obtained from the jugular vein (or other peripheral vein) prior to vaccination. Blood is collected into a 12 mL serum separator tube and allowed to clot.

Blood is centrifuged, serum removed and aliquoted into two plastic shipping tubes (Replicate A and B). Serum samples are stored frozen prior to shipment. The plastic tubes are labeled with the animal identification number, replicate ID and the date of collection. Laboratory submission forms (supplied by laboratory) and chain-of-custody forms are completed for each sample submission. Frozen samples (Replicate A) are shipped, on ice, weekly to a predetermined laboratory. Upon receipt confirmation of the primary sample, retainer samples (Replicate B) will also be shipped on ice to the participating laboratory. The laboratory are masked to the animal's vaccination history. Dates of blood sample collection and vaccination are recorded.

One laboratory is identified to analyze all serologic samples. The laboratory is required to standardize and validate all assays in order to meet regulatory requirements.

d. Treatment Administration

On Day 0, after collection of pre-vaccination serum samples, dogs are administered one dose (1 mL) of Vanguard Plus® 5/L as per label directions.

e. Post-Vaccination Blood Collection and Processing

Three to 7 days (Study Day 4 - 6) following vaccination, the dog is re-examined and a second blood sample obtained and processed (as described above for pre-vaccination samples) and recorded. Once the post-vaccination sample is collected, the dog is considered off the study.

f. Serological Titers

The following antibody titers are determined for each serum sample: canine distemper virus serum neutralizing ("SN") titer; canine parvovirus SN titer; canine adenovirus type 1 SN titer; canine adenovirus type 2 SN titer; canine parainfluenza virus SN titer; *Leptospira interrogans* ser. *icterohaemorrhagiae* microscopic agglutination ("MA") titer; *Leptospira interrogans* ser. *canicola* MA titer.

The priority for sample analysis is as follows:

1. Canine distemper virus SN titer;
2. Canine parvovirus SN titer;
3. Canine adenovirus type 1 SN titer;
4. Canine adenovirus type 2 SN titer;
5. *Leptospira interrogans* ser. *icterohaemorrhagiae* MA titer;
6. *Leptospira interrogans*, ser. *canicola* MA titer;
7. Canine parainfluenza virus SN titer.

g. Blood Collection and Processing

1. Collect approximately 10 mL of blood into a serum separator (red top) vacutainer tube. Blood can be collected either by syringe, or directly into the vacutainer tube.
2. Allow blood to clot a minimum of 20 minutes, but no more than 40 minutes.

3. Centrifuge clotted blood for a minimum of 5 minutes at appropriate speed.
4. Remove red rubber stopper from separator tube, and carefully pour or pipette serum in two approximately equal aliquots into two plastic shipping tubes. Close the plastic shipping tubes with the cap provided, and apply labels with animal name, animal identification number, and date of collection. Freeze samples at approximately -20°C. If a pipette is used to transfer serum, a new pipette should be used for each sample.
5. Blood should be processed and frozen within two hours of collection.

h. Concurrent Disease/Medication

If medication, medical or surgical procedures are deemed necessary, then standard veterinary practice procedures are followed. No concurrent medications (other than defined permissible concurrent medications), medical or surgical procedures are permitted without prior consent of the Study Director, with the exception of extreme emergencies when even short delays would be life-threatening or cause undue suffering. Animals can be removed from the study, if warranted.

Dogs can be treated with the following medications during the course of the study with the use documented to include drug, dosage, duration and reason for use:

1. Daily or monthly heartworm preventative (e.g., selamectin, diethylcarbamazine, ivermectin, milbemycin)
2. Monthly oral flea preventative (e.g., lufenuron)
3. Topical flea treatment (e.g., selamectin, imidacloprid, organophosphate or pyrethroid based sprays, dips, or powders)
4. Prescription diets
5. Oral antihelminthics (e.g., pyrantel, albendazole, fenbendazole)
6. Medications to control underlying medical disorders (e.g., antimicrobials, thyroid supplementation) can be used if use is documented to identify drug, dosage, duration, dates and reason for use.

6. DATA MANAGEMENT AND STATISTICAL ANALYSIS:

Animals are categorized into groups based on the period of time between their last vaccination and their entry into the study as follows:

- 12-18 months
- 19-24 months
- 25-30 months, etc.

An animal is put into one of 4 risk categories based on the Lifestyle and Exposure Risk Questionnaire. The categories are low exposure to other dogs and low exposure to other disease vectors (low-low), low exposure to other dogs and high exposure to other disease vectors (low-high), high exposure to other dogs and low exposure to other disease

vectors (high-low), and high exposure to other dogs and high exposure to other disease vectors (high-high).

- In this application the term "high risk animal", whether applied to dogs, cats or another animal, applies to any animal meeting the criteria for inclusion in either the high-low or high-high categories, while the term "low risk animal" applies to any animal meeting the criteria for inclusion in either the low-low or low-high categories".

a. Lifestyle and Exposure Risk Questionnaire

1. Which of the following best describes the area where you and your dog live? (check one) <input type="checkbox"/> city <input type="checkbox"/> suburb <input type="checkbox"/> town, village, or hamlet <input type="checkbox"/> farm or ranch
2. In years, how long have you lived in your present home?
3. In total, how many dogs are in your household? (If there is only one dog in your household, go to question 4.)
3a. How many of these dogs are one year of age or older?
3b. How many of these dogs are less than one year of age?
3c. Have any new dogs been acquired during the last year?
4. During the past year, did you take your dog outdoors to exercise, urinate or defecate? If the answer is no, go to question 5. <input type="checkbox"/> yes <input type="checkbox"/> no
When you took/let your dog outdoors, did you: 4a. Confine your dog to a kennel, fenced area, or yard? <input type="checkbox"/> yes <input type="checkbox"/> no
4b. Tether your dog to a run, tree, post, or stake? <input type="checkbox"/> yes <input type="checkbox"/> no
4c. Walk your dog on a leash? <input type="checkbox"/> yes <input type="checkbox"/> no
4d. Allow your dog to roam free with supervision ( <i>i.e.</i> , your dog is visible at all times)? <input type="checkbox"/> yes <input type="checkbox"/> no



4e. Allow your dog to roam free without supervision? <input type="checkbox"/> yes <input type="checkbox"/> no
4f. Walk or exercise your dog in a park, field, or other area where other dogs are walked or exercised? <input type="checkbox"/> yes <input type="checkbox"/> no
During the past year, when your dog was outdoors, did it: 4g. Walk or exercise or play with other dogs in your household? <input type="checkbox"/> yes <input type="checkbox"/> no
4h. Walk or exercise or play with other dogs not in your household? <input type="checkbox"/> yes <input type="checkbox"/> no
4i. Ever fight, kill, or share food or water bowls with a wild animal? <input type="checkbox"/> yes <input type="checkbox"/> no
4j. Ever drink from puddles of water, ponds, streams, rivers, or lakes?? <input type="checkbox"/> yes <input type="checkbox"/> no
4k. Ever swim in ponds, streams, rivers, or lakes? <input type="checkbox"/> yes <input type="checkbox"/> no
4l. Ever eat garbage, refuse, animal carcasses, or trash? <input type="checkbox"/> yes <input type="checkbox"/> no
4m. Ever eat another dog's feces? <input type="checkbox"/> yes <input type="checkbox"/> no
5. Did you ever take dog to a dog show or field trials? <input type="checkbox"/> yes <input type="checkbox"/> no
6. Did you ever take your dog to obedience classes? <input type="checkbox"/> yes <input type="checkbox"/> no
7. Did you ever take your dog to a boarding kennel? <input type="checkbox"/> yes

<input type="checkbox"/> no
8. Did you ever have your dog groomed by a professional groomer?
<input type="checkbox"/> yes
<input type="checkbox"/> no

5 An animal is put in the low-low risk category if the answer to question 3 is "1", the answer to question 4 is "No" and the answers to questions 5,6, 7 and 8 are "No" or if the answer to question 3 is "1", the answer to question 4 is "Yes", the answer to at least one of questions 4A, 4B or 4C is "Yes" and the answers to questions 4D, 4E, 4F, 4G, 4H, 4I, 4J, 4K, 4L, 4M, 5,6,7 and 8 are "No".

10 An animal is classified as low-high if the answer to question 3 is "1", the answer to question 4 is "Yes", the answer to at least one of questions 4A, 4B or 4C is "Yes", the answers to questions 4D and 4E are "No", the answer to question 4F is "Yes", the answers to questions 4G and 4H are "No", the answer to at least one of questions 4I, 4J, 4K, 4L and 4M is "Yes" and the answers to questions 5,6,7 and 8 are "No".

15 An animal is put in the high-low category if the answer to question 3 is "1", then answer to question 4 is "No", the answer to at least one of questions 5,6,7 or 8 is "Yes", or if the answer to question 3 is "> 1" the answer to question 4 is "Yes", the answer to at least one of questions 4A, 4B or 4C is "Yes", the answers to questions 4D, 4E and 4F is "No", the answer to at least one of questions 4G, 4H, 5, 6,7 or 8 is "Yes" and the answers to questions 4I, 4J, 4K, 4L and 4M are "No".

20 The animal is classified as high-high if the answer to question 3 is ">1", the answer to question 4 is "Yes", the answer to at least one of questions 4D and 4E is "Yes", the answer to question 4F is "Yes", the answer to at least one of questions 4G, 4H, 5, 6, 7 and 8 is "Yes", and the answer to at least one of questions 4I, 4J, 4K, 4L and 4M is "Yes".

#### 7. Determining Whether the Animal is Immune

25 In regions where the clinic records indicate active disease prevalence (or local diagnostic lab data confirms active disease cases), animals that are classified as other than low risk and which have no history of the disease of interest are also to be considered immune (this being considered evidence of immunity in that they remained healthy even when exposed to disease in the environment.) In regions of low (or zero) disease prevalence animals of any risk level will be assigned immune/not immune status based upon the residual post vaccination antibody titers and anamnestic responses.

30 An animal is considered to be immune to a specific disease if the animal has antibody levels indicating an ongoing immune response for that disease at the time of entry into the study and/or an anamnestic response is induced for that disease by 3-7 days post-vaccination

and the animal has no previous history of having that disease. Antibody titers that indicate an ongoing immune response are defined as follows:

- a) Canine distemper virus SN titer  $\geq 1:16$
- b) Canine parvovirus SN titer  $\geq 1:16$
- 5 c) Canine adenovirus, type 1 SN titer  $\geq 1:16$
- d) Canine adenovirus, type 2 SN titer  $\geq 1:16$
- e) Canine parainfluenza Virus SN titer  $\geq 1:4$
- f) *Leptospira interrogans*, ser. *icterohaemorrhagiae* MA titer  $\geq 1:8$
- g) *Leptospira interrogans* ser. *canicola* MA titer  $\geq 1:8$

10 An anamnestic response is defined as an increase in antibody titers of two or more doubling dilutions (four-fold increase) between the blood sample obtained before revaccination and the blood sample obtained 3 - 7 days after revaccination.

The relationship between pre-vaccination time and immune/not immune status is used to assess duration of adequate immune memory for each disease using the logistic regression model discussed above. Only animals with both the first and second blood  
15 samples are included in the analysis. Estimates from the logistic regression model are used to determine the duration of adequate immune memory for each antigen as defined as the predicted prevaccination time-point where the percentage of immune animals is  $\geq 80\%$ .

20 8. Adverse Experience Reporting:

All dogs entered into the study and administered vaccine are observed by the veterinarian and pet owner for the occurrence of any abnormal clinical signs during the following 10 days.

The Study Director will document any telephone reports of unexpected or abnormal  
25 clinical signs made by owner/agents and will, based on professional judgement, determine if the abnormal clinical signs observed warrant an unscheduled examination of the animal.

In the unlikely event that an animal should die for any reason during the course of the study, a necropsy should be conducted by a veterinarian or a veterinary pathologist to determine the cause of death.

30 EXAMPLE 2: A prospective study to determine the duration of adequate immune memory of the various viral and bacterial components of Felocell® CVR (generic name: feline panleukopenia-herpesvirus-calicivirus, modified live virus vaccine) in cats by measuring antibody titers, disease incidence, and assessing risk of exposure to disease over time.

1. Study Animals:

35 Species	Feline	Type:	Any
Breed/Strain:	Any	Sex:	Male or Female (intact or neutered)
Description:	Adults		

Origin: Privately owned veterinary patients

Initial Age: Min: 2 Max: 3 Units: Years

Weights: Min: None Max: None Units: Pounds

## 2. MANAGEMENT:

### 5 a. Site Specifications

This study is a controlled clinical trial conducted at multiple sites over diverse geographic regions in the United States and Canada. One protocol common to all sites is used. Approximately 80-100 veterinary practices will participate in the trial. Each individual study site is expected to enroll a minimum of 25 to 30 cats.

10 The sites are small-animal veterinary practices whose clientele, record keeping and compliance will provide a large proportion of enrolled study animals that satisfactorily complete the study. The owners of the cats shall be willing to participate in the study until completion of all observations and collection of all samples. The practices should have at least one veterinarian (the Study Director or Examining Veterinarian designated by the Study  
15 Director) who is experienced in general veterinary practice and medicine.

The practices will have a history of using Felocell® CVR over the last three years.

### b. Pretreatment

Medical management will follow normal accepted clinical practice for each study animal. The use of concomitant medications is recorded.

### 20 c. Identification Method

Individual animal identification is provided by owners and unique case report numbers.

### d. Housing

25 Cats are kept in normal domestic environments and can be housed either in the client's house or in separate animal accommodation. Cats can be housed with or without other animals.

### e. Feeding and Watering Methods

Normal practice.

## 3. STUDY GROUPS

30 Cats are selected for the study from animals presented to each veterinary practice such that different risk groups are represented. The medical records of each site are reviewed to identify adult cats that satisfy all of the inclusion criteria and for which none of the exclusion criteria apply.

Animals are assigned to study groups T01 or T02. Animals selected for study group  
35 T01 will serve as positive controls and will receive vaccinations annually based on current label recommendations for Felocell® CVR. Animals selected for study group T02 will not be re-vaccinated, but will have antibody titers and incidence of disease monitored throughout the

study. On Day 0, disease history and disease risk analysis are recorded and a complete physical examination performed for each animal. Also on Day 0, cats in T01 are administered one dose of Felocell® CVR subcutaneously as per label directions.

No.	Time Since Last Vaccination	Route	Vaccination Schedule	Animals
T01	Approx. 12-24 months	Subcutaneous	annually	Approx. 500
T02	Approx. 12-24 months	N/A	N/A	Approx. 1000

5 a. Inclusion Criteria For Study Group T01

1. Any breed, sex or weight of cat.
2. Healthy cats, 2 – 3 years of age.
3. Cat has been vaccinated with Felocell® CVR within the past 11-13 months. Cats may have received rabies, Feline Leukemia, Feline Infectious Peritonitis or *Bordetella* vaccinations within the past 12-months.
4. Cat must have a history of vaccination with a minimum of two doses of a Felocell® CVR given as a kitten as a priming dose, with the last dose administered when the animal is 12- 16 weeks of age or older.
5. Cat must have received at least one additional dose of Felocell® CVR 9 - 14 months after the initial priming series.
6. The name product types and serial numbers of the Felocell® CVR vaccines used and the dates of vaccination must be recorded in the study animal medical record.
7. The owner/authorized agent must agree to return the study animal to the veterinary practice for evaluation and sample collection at the required times.
8. The owner/authorized agent must give written informed consent for the animal to participate in the study.
9. The owner must complete the disease history and disease risk analysis questionnaire at each scheduled visit or by phone (Appendix IV, Form 7).

25 b. Inclusion Criteria For Study Group T02

1. Any breed, sex or weight of cat
2. Healthy cats, 2 -3 years of age.
3. Cat has not been vaccinated with Felocell® CVR within the past 12-24 months. Cat must not have received a mono- or polyvalent vaccine containing feline panleukopenia-herpesvirus-calicivirus and *Chlamydia psittaci* within the past 11 – 25 months. Cats may have received rabies, Feline Leukemia, Feline Infectious Peritonitis or *Bordetella* vaccinations within the past 12-months.

4. Cat must have a history of vaccination with a minimum of two doses of a Felocell® CVR given as a kitten as a priming dose, with the last dose administered when the animal is 12 – 16 weeks of age or older.
5. Cat must have received at least one additional dose of Felocell® CVR, 9 - 14 months after the initial priming series.
6. The name product types and serial numbers of the Felocell® CVR vaccine used and the dates of vaccination must be recorded in the study animal medical record.
7. The owner/authorized agent must agree to return the study animal to the veterinary practice for evaluation and sample collection at the required times.
- 10 8. The owner/authorized agent must give written informed consent for the animal to participate in the study.
9. The owner must complete the risk analysis questionnaire at each scheduled visit or by phone.

c. Exclusion Criteria for All Cats

- 15 1. Cats with a history of intolerance/allergy to vaccinations.
2. Pregnant animals.
3. Cats with severe systemic disease, whether or not it is a constant threat to life (renal failure, hepatic failure, terminal neoplasia).
4. Cats treated with any immunosuppressive drugs (corticosteroids including prednisone, and dexamethasone, or other immunosuppressives such as cyclophosphamide, azathioprine, and gold sodium thiomalate, etc.) within the past 10 days.
- 20 5. Cats treated with repository anti-inflammatory drugs (e.g., methyl-prednisolone acetate) within the last 30 days.
6. Cats that are FeLV or FIV positive on ELISA test.

25

4. RISK ANALYSIS QUESTIONNAIRE

Animals are placed into one of two risk categories based on the lifestyle and disease according to the following questions answered by the pet owner.

1. Which of the following best describes the area where you and your cat live? (check one) <input type="checkbox"/> city <input type="checkbox"/> suburb <input type="checkbox"/> town, village, or hamlet <input type="checkbox"/> farm or ranch
2. In years, how long have you lived in your present home?
3. In total, how many cats are in your household? (If there is only one cat in your household, go to question 4.)
3a. How many of these cats are one year of age or older

3b. How many of these cats are less than one year of age?
3c. Have any new cats been acquired during the last year?
4. Which of the following best describes the housing of your cat(s)? (check one) <input type="checkbox"/> All cats are indoors 100% of the time <input type="checkbox"/> One or more cats go outside but not every day <input type="checkbox"/> One or more cats goes outside every day <input type="checkbox"/> One or more cats are outdoors 100% of the time
5. Which of the following best describes the housing of the cat here today?(check one) <input type="checkbox"/> All cats are indoors 100% of the time <input type="checkbox"/> One or more cats go outside but not every day <input type="checkbox"/> One or more cats goes outside every day <input type="checkbox"/> One or more cats are outdoors 100% of the time
6. Did you board your cat in a boarding kennel any time during the last year?(check one) <input type="checkbox"/> yes <input type="checkbox"/> no
7. Did your cat ever escape from the house and was lost or missing for more than 24 hours?(check one) <input type="checkbox"/> yes <input type="checkbox"/> no
8. Did you take your cat to cat shows?(check one) <input type="checkbox"/> yes <input type="checkbox"/> no

An animal is placed in the low-risk category if the answer to question 3 is "1", the answer to question 5 is "100% indoors" and the answers to questions 6, 7, and 8 are "No" or if the answer to question 3 is "2" or "3", the answer to question 3c is "No", the answers to questions 4 and 5 are "100% indoors" and the answers to questions 6, 7, and 8 are "No". Otherwise the animal is categorized as being in the high-risk category. The risk category is determined for every time a questionnaire is completed. For purposes of statistical analysis, once an animal is categorized as high in either the exposure to other cats or exposure to other disease vectors it cannot be reclassified as low in that category.

## 10 5. PROCEDURES

### a. Examinations, Serum Collection and Vaccination for the T01 Study Group

Following initial examination on the enrollment date, owners of cats in study group T01 are interviewed in person or by phone at 6 month ( $26 \pm 2$  weeks) intervals for the duration of the study to determine disease history and conduct disease risk analysis. Disease history and disease risk analysis will occur in person at the first 6-month exam and again at the 1-

year exam because cats are required to visit the participating clinic for blood sample collection to determine serum antibody titers. Blood samples will not be collected beyond year one from cats in T01. The cats are administered one dose of Felocell® Plus® 5/L subcutaneously as per label directions at each 12-month ( $52 \pm 2$  weeks) interval for the duration of the study.

b. Examinations and Serum Collection for the T02 Study Group

For cats in the T02 study group, on the enrollment date, disease history and disease risk analysis are recorded and a physical examination is performed for each animal. Also on the enrollment date, (once the cat is confirmed to meet inclusion criteria), approximately, five (5) mL of whole blood will be obtained from the jugular (or other peripheral) vein.

Cats in the T02 study group are then re-examined at 6-month ( $26 \pm 2$  weeks) intervals following the initial examination for the duration of the study. At each 6-month visit, disease history and disease risk analysis will be recorded, a physical examination performed, and a blood sample collected for the determination of serum antibody titers. When serum antibody titers drop below defined levels for any one antigen, the animal will receive a monovalent vaccine containing that antigen only.

c. Serum Sample Processing

On the enrollment date, approximately, five (5) mL of blood is obtained from the jugular (or other peripheral vein) prior to vaccination. One half (0.5) mL of whole blood is used to test for the presence of FELV antigen and FIV antibody using a standard combination ELISA test kit and the results recorded on the animal physical examination form. The remainder of the blood is collected into a 7 mL serum separator tube (red top) and allowed to clot. If the results of the FELV and FIV ELISA are negative and the cat is confirmed to meet the inclusion criteria, then blood samples are centrifuged, then the serum is removed and aliquoted into two plastic shipping tubes (Replicates A and B). All serum samples are stored frozen prior to shipment. The plastic tubes are labeled with the animal identification number, replicate ID and the date of collection. Laboratory submission forms (supplied by laboratory) and chain-of-custody forms are completed for each sample submission.

Frozen samples (Replicate A) are shipped, on dry ice, weekly, to a predetermined laboratory. Upon receipt confirmation of the primary sample, retainer samples (Replicate B) will also be shipped on ice to the participating laboratory. The laboratory is masked to the animal's vaccination history. Dates of blood sample collection and vaccination are recorded.

One laboratory is identified to analyze all serologic samples. The laboratory will standardize and validate all assays to meet regulatory requirements prior to initiation of sample analysis.

d. Blood Collection And Processing



1. Collect approximately 5 mL of blood into a serum separator (red top) vacutainer tube. Blood can be collected either by syringe, or directly into the vacutainer tube.
2. Allow blood to clot a minimum of 20 minutes, but no more than 40 minutes.
3. Centrifuge clotted blood for a minimum of 5 minutes at appropriate speed.
- 5 4. Remove red rubber stopper from separator tube, and carefully pour serum into plastic shipping tube. Close the plastic shipping tube with the cap provided, and apply label with animal name, animal identification number, and date of collection. Freeze sample. If a pipette is used to transfer serum, a new pipette should be used for each sample.
5. Blood should be processed and frozen within two hours of collection.

10 e. Serological Titers

The following antibody titers are determined for each serum sample: feline panleukopenia virus serum neutralizing (SN) titer; feline herpesvirus SN titer; feline calicivirus SN titer; *Chlamydia psittaci* microscopic agglutination (MA) titer. The laboratory will retain all remaining sera until notice from the Study Monitor is given.

15 The priority for sample analysis is as follows:

1. feline panleukopenia virus SN titer;
2. feline herpesvirus SN titer;
3. feline calicivirus SN titer;
4. *Chlamydia psittaci* MA titer

20 f. Revaccination of T02 Cats

Laboratory results of serum titers for each are available to the veterinarian 7-10 working days following vaccination. The serum antibody levels are used to determine if additional vaccines need to be administered. When the serum antibody titer for a viral or bacterial antigen decreases below the minimum level that indicates an immune response, the owner is contacted by phone and advised to have the cat revaccinated. The owner will return the cat to be revaccinated with a monovalent vaccine containing that particular antigen. Once revaccinated, the cat will remain in the study, subsequent serum antibody titers measured for the revaccinated antigen will not be included in the analysis.

g. Concurrent Disease/Medication

30 Cats can be treated with the following medications during the course of the study with the use documented to include drug, dosage, duration and reason for use:

1. Monthly heartworm preventative (e.g., selamectin, ivermectin)
2. Monthly oral flea preventative (e.g., lufenuron)
3. Topical flea treatment (e.g., selamectin, imidacloprid or pyrethroid based sprays, dips, or powders)
- 35 4. Prescription diets
5. Oral antihelminthics (e.g., pyrantel, albendazole, fenbendazole)

6. Medications to control underlying medical disorders (e.g., antimicrobials, insulin, methimazole) can be used if use is documented to identify drug, dosage, duration, dates and reason for use.

h. Termination of Data Collection

5       Animals in study groups T01 and T02 are followed for approximately 4 years after entry into the study. Data collection is terminated for a particular antigen when (80%) of the T02 study population no longer maintains antibody titers suggestive of an active immune response. Early termination of the study will occur when (80%) of the T02 study population no longer maintains antibody titers indicating an active immune response against feline  
10       panleukopenia virus.

6. **ADVERSE EXPERIENCE REPORTING:**

      All cats entered into the study and administered vaccine are observed by the veterinarian and pet owner for the occurrence of any abnormal clinical signs during the following 10 days.

15       The Study Director will document any reports of unexpected or abnormal clinical signs made by owner/agents and will, based on professional judgement, determine if the abnormal clinical signs observed warrant an unscheduled examination of the animal.

      In the event that an animal should die for any reason during the course of the study, a necropsy should be conducted by a veterinarian or a veterinary pathologist to determine the cause of death, if possible. Where appropriate, at the discretion of the veterinary pathologist, samples are collected for histopathological and/or microbiological examination and a thorough report of macroscopic and microscopic findings are completed and reported.

7. **DATA MANAGEMENT AND STATISTICAL ANALYSIS**

25       In regions where the clinic records indicate active disease prevalence (or local diagnostic lab data confirms active disease cases) animals that are classified as other than low risk and which have no history of the specific disease of interest are also to be considered immune T(his being considered evidence of immunity in that they remained healthy even when exposed to the specific disease in the environment.) In regions of low (or zero) disease prevalence, animals of any risk level will be assigned immune/not immune status based upon  
30       the antibody titers and responses.

      An animal is considered to be immune to a specific disease if the animal has detectable antibody levels for that disease at the time of blood sampling and no previous history of having that disease. Otherwise, the animal is categorized as not immune. Preferably, the antibody titers that indicate an ongoing immune response are as follows:

- 35       a)       feline panleukopenia virus SN titer  $\geq 1:16$ ;  
      b)       feline herpesvirus SN titer  $\geq 1:2$ ;  
      c)       feline calicivirus SN titer  $\geq 1:4$ ;

d) *Chlamydia psittaci* MA titer  $\geq 1:8$ .

If an animal remains immune throughout the study or is immune while it remains in the study, it is recorded as censored and the time until no longer immune recorded as its last available time point. The duration of adequate immune memory is estimated by a duration of

5 adequate immune memory prediction equation as described above.

# SCHEDULE OF OPERATIONS

## T01 Vaccinated Control Cats

WEEK OF STUDY	EVENT/ACTIVITY
0	Physical exam, blood sample, vaccinate, disease history, risk evaluation
26 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
52 $\pm$ 2	Physical exam, blood sample, vaccinate, disease history, risk evaluation
78 $\pm$ 2	Physical exam, disease history, risk evaluation
104 $\pm$ 2	Physical exam, vaccinate, disease history, risk evaluation
130 $\pm$ 2	Physical exam, disease history, risk evaluation
156 $\pm$ 2	Physical exam, vaccinate, disease history, risk evaluation
182 $\pm$ 2	Physical exam, disease history, risk evaluation
208 $\pm$ 2	Physical exam, vaccinate, disease history, risk evaluation
234 $\pm$ 2	Physical exam, disease history, risk evaluation

10

## T02 Non-vaccinated Study Cats

WEEK OF STUDY	EVENT/ACTIVITY
0	Physical exam, blood sample, disease history, risk evaluation
26 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
52 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
78 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
104 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
130 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
156 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
182 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
208 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation
234 $\pm$ 2	Physical exam, blood sample, disease history, risk evaluation

Although the present invention has been described in considerable detail with reference to certain preferred embodiments and while it is apparent that the invention

[illegible]